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Lab Resource: Stem Cell Line

Derivation of the human embryonic stem cell line RCe009-A (RC-5)

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ABSTRACT

The human embryonic stem cell line RCe009-A (RC-5) was derived from a frozen and thawed Day 2 embryo voluntarily donated as unsuitable and surplus to requirement for fertility treatment following informed consent under licence from the UK Human Fertilisation and Embryology Authority. RCe009-A carries the common DF508 mutation on the cystic fibrosis trans-membrane regulator gene associated with the disease cystic fibrosis. The cell line shows normal pluripotency marker expression and differentiation to the three germ layers *in vitro*. It has a normal 46XX female karyotype and microsatellite PCR identity, HLA and blood group typing data are available.

Resource Table

Name of stem cell construct	RCe009-A
Alternative name	RC-5, RC5
Institution	Roslin Cells Ltd.
Person who created resource	B. Tye, K. Bruce, P. Dand, G. Russell, D. M. Collins, J. Gardner
Contact person and email	Paul.desousa@roslincells.com; Paul.desousa@ed.ac.uk Janet.downie@roslincells.com Aidan.courtney@roslincells.com Malcolm.bateman@roslinfoundation.com
Date archived/stock date	14 March 2008
Type of resource	Biological reagent: cell line
Sub-type	hESC, research grade
Origin	Cleavage stage embryo cultured to blastocyst stage.
Key transcription factors	Oct4 and Nanog, (confirmed by immunocytochemistry)
Authentication	See Quality Control Test Summary, Table 1
Link to related literature (direct URL links and full references)	N/A
Information in public databases	http://hpscreg.eu/cell-line/RCe009-A
Ethics	Informed consent obtained. Scotland A Research Ethics committee approval obtained (07/MRE00/56). Conducted under the UK Human Fertilisation and Embryology Authority licence no R0136 to centre 0202.

Resource Details

RCe009-A (RC-5) was derived from a frozen and thawed, pre-implantation genetic diagnosis embryo confirmed to have a heterozygous cystic fibrosis mutation (DF508 on the CTFR gene). The embryo was received as a Day 2 embryo and grown to blastocyst stage. The cell line was derived by whole embryo outgrowth on mitotically inactivated human fibroblast (HDF) feeder cells using HDF conditioned medium and expanded under feeder free conditions.

RCe009-A (RC-5) was shown to be pluripotent by expression of the pluripotency markers Oct-4, Nanog, SSEA-4, Tra-1-60 and Tra-1-81, but not the differentiation marker SSEA-1, using immunocytochemistry (Table 1, Fig. 1). By flow cytometric analysis, the expression of pluripotency makers Tra-1-60, Tra-1-81 and SSEA-4 was 94.8, 93.6% and 94.8%, respectively, but some expression of the differentiation marker SSEA-1 (37.3%) was observed (Fig. 2). Differentiation to the three germ layers, endoderm, ectoderm and mesoderm, was demonstrated using embryoid body formation and expression of the germ layer markers α-fetoprotein, β-tubulin and muscle actin (Fig. 3).

A microsatellite PCR profile has been obtained for the cell line, and HLA Class I and II typing is available (Table 2). Blood group genotyping gave the blood group AO_1 (Table 2).

Verification and authentication

The cell line was analysed for genome stability by G-banding (Fig. 4) and showed a normal 46XX female genotype. The cell line is free from mycoplasma contamination as determined by RC-qPCR. Microsatellite PCR DNA profiling for cell identity is available.

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Table 1
Summary of quality control testing and results for RC-5 (RCe009-A).

Classification	Test	Purpose	Result
Donor screening	HIV 1 + 2 Hepatitis B Hepatitis C	Donor screening for adventitious agents	Negative
Identity	Microsatellite PCR (mPCR)	DNA Profiling to give cell line its signature, gender/species	Performed
Phenotype	Immunocytochemisty	To assess levels of staining for the pluripotency markers	Expression of Oct4, Nanog, SSEA-4, Tra-1-60 and Tra-1-81
	Flow cytometry	Assess antigen levels and cell surface markers commonly associated with hESC	Tra-1-60: 94.8% Tra-1-81: 93.6% SSEA-4: 94.8% SSEA-1: 37.3%
Genotype (details provided in Table 2)	Blood group genotyping (DNA analysis)	To establish blood group of the line	AO_1
	Karyology (G-banding)	Confirmation of normal ploidy by G-banding	46XX
	HLA tissue typing	To establish full HLA Type I and II genotype of the line	HLA typed Class I and Class II
Microbiology and Virology	Mycoplasma	Mycoplasma testing by RT-qPCR	Negative
	Endotoxin	Screening for endotoxin levels	1.82 EU/mL
Morphology	Photography	To capture a visual record of the line	Normal
Differentiation potential	Embryoid body formation	To show differentiation to three germ layers	Expression of muscle actin, β-tubulin and α-fetoprotein

Materials and methods

Ethics

Derivation of hESC from surplus to requirement and failed to fertilise/develop embryos was approved by The Scotland A Research Ethics Committee and local ethics board at participating fertility clinics and conducted under licence no. R0136 from the UK HFEA with informed donor consent.

Cell culture

Frozen embryos were thawed using Embryo Thawing Pack (Origio (Medicult), Denmark) using standard techniques and were cultured in EmbryoAssist (Origio) until Day 3 and BlastAssist (Origio) after Day 3 of development. Embryos were cultured at 36.5–37.5 °C, 5 ± 0.5% CO_2, 5 ± 0.5% O_2 in drops under paraffin oil (Origio) and transferred to fresh medium at least every 2–3 days.

By Day 8 of development, or when spontaneous hatching occurred, embryos were placed in derivation conditions consisting of mitotically inactivated neonatal human dermal fibroblasts (HDFs) (ThermoFisher Scientific (Cascade Biologics), Paisley, UK) on tissue culture plastic precoated with 2 μg/cm^2 human laminin (Sigma Aldrich, Dorset, UK) as per manufacturer's recommendation. If required, assisted hatching was performed by removing the zona pellucidae mechanically using Swemed Cutting tools (Vitrolife, Göteborg, Sweden).

HDF cells were cultured in DMEM (Lonza, Slough, UK), 10% FCS (GE Healthcare (PAA), Buckinghamshire, UK) and 2 mM L-glutamine (ThermoFisher Scientific). HDF were mitotically inactivated using gamma irradiation at 50GY using a Gammacell Elite 1000 machine. For use as a feeder layer, irradiated HDFs were plated at 2–50,000 cells/cm^2 in HDF conditioned medium (80% Knockout-DMEM, 20% Knockout serum replacement (KOSR), 1 mM glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 4 ng/ml human bFGF (all ThermoFisher Scientific) over 24 h intervals over 7 days) supplemented with an additional 24 ng/ml human bFGF. Cells were cultured at 36.5–37.5 °C, 5 ± 0.5% CO_2, 5 ± 0.5% O_2 and 50% medium exchanged 6 days a week.

The established cell line was expanded and banked using CellStart matrix and Stempro hESC Serum Free Medium (ThermoFisher

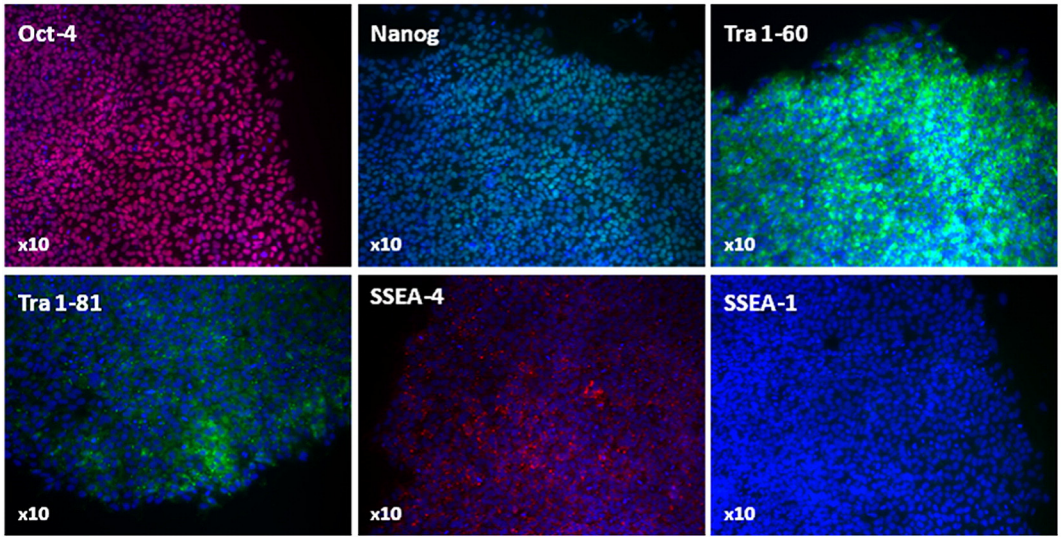

Fig. 1. Immunostaining of RCe009-A (RC5) show expression of pluripotency markers Oct-4, Nanog, Tra-1-60, Tra-1-81 and SSEA-4, but not differentiation marker SSEA-1.

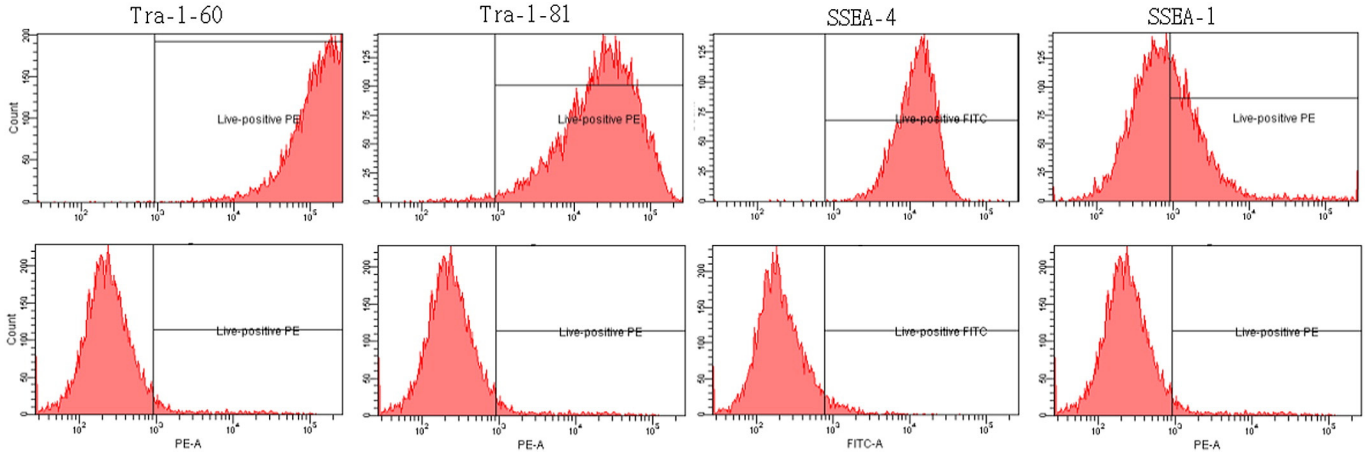

Fig. 2. RCe009-A (RC-5) was subjected to flow cytometry analysis for markers of pluripotency with isotype controls (left hand column) or specific antibodies for SSEA-1, Tra-1-60 and Tra-1-81 (top row) or SSEA-4 (bottom row). Percentage staining is indicated in Table 1.

Scientific). Passaging was performed mechanically using an EZ passage tool (ThermoFisher Scientific). hESC lines were expanded to 25–30 wells of a 6-well plate and cryopreserved in 0.5-1 ml KOSR based cryopreservation solution (75% KO-DMEM, 15% Xeno-free KOSR (ThermoFisher Scientific) and 10% DMSO (Origen Biomedical, Texas, USA)) or Cryostor CS10 (Biolife Solution, Washington, USA).

Mycoplasma

Mycoplasma detection was performed using Applied Biosystems PrepSEQ™ Mycoplasma Nucleic Acid Extraction Kit and MicroSEQ™ Mycoplasma Real-Time PCR Detection Kit (ThermoFisher Scientific (Applied Biosystems)) according to manufacturer's instruction.

Endotoxin

Endotoxin levels were determined using the Kinetic-QCL assay (Lonza) and an incubating plate reader (BioTek ELx808) according to manufacturer's instructions. Briefly, an unknown sample was compared with a standard curve of known levels of control endotoxin. An assay was deemed valid if the coefficient of correlation, $r \geq 0.980$ and the CV (%) for the standard curve was ≤10%.

Flow cytometry

Human embryonic stem cells were dissociated using Trypsin (ThermoFisher Scientific). Non-specific staining was blocked using 5% goat serum (Sigma) in PBS (Lonza) containing 0.01% Tween-20 (Sigma). Cells were stained with antibodies against SSEA-4, SSEA-1, Tra-1-60 and Tra-1-81 (all BD, Oxford, UK), at 250 ng per reaction followed by Goat F(ab)2 anti-mouse IgM-PE Goat F(ab)2 anti-mouse IgG3-FITC (1:200; Santa Cruz Biotechnology, Texas, USA). Cells were analysed using a FACS Aria flow cytometer (BD).

Immunocytochemistry

hESC were fixed in 4% paraformaldehyde (ThermoFisher Scientific (Alfa Aesar)), permeabilised using 100% ethanol (ThermoFisher Scientific) and stained with AFP (1:500; Sigma), β-tubulin III (1:1000; Sigma), muscle-specific actin (1:50; DAKO, Glostrup, Denmark), Oct-4 (1:200; Santa Cruz Biotechnlogy, Texas, USA), Nanog (1:20; R&D Systems, Abingdon, UK), Tra-1-60, Tra-1-81, SSEA-1 and SSEA-4 (all 1:50; BD) and secondary antibodies anti-mouse IgG-FITC (1:200; Sigma), anti-mouse IgG-AlexaFluor 488, anti-goat IgG-AlexaFluor 488, anti-goat IgG-AlexaFluor-594 and anti-donkey polyclonal AlexaFluor-594 (all 1:200; ThermoFisher Scientific). Images were acquired using a Zeiss S100 Axiovert fluorescence microscope or Nikon eC1 confocal microscope.

In vitro differentiation

hESC cells were pre-treated for 1 h with 10 μM ROCK inhibitor in Stempro hESC SFM (ThermoFisher Scientific) and embryoid bodies EBs generated in ultra low attachment plates (Corning) for 7 days before being transferred into EB medium (20% FBS (GE Healthcare (PAA)), 80% KO-DMEM 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids (all ThermoFisher Scientific)), on glass slide tissue culture chambers (Nunc, ThermoFisher Scientific) coated with 0.5% gelatin (Sigma) at 0.1 ml/cm^2 for 14 days.

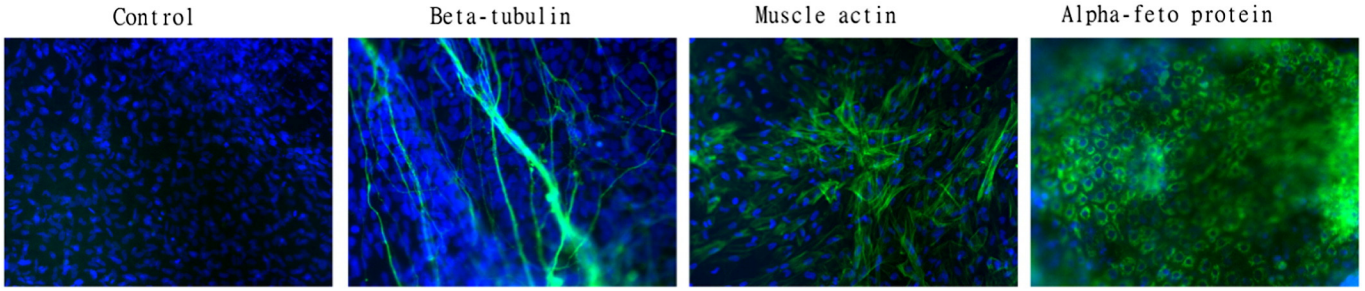

Fig. 3. In vitro differentiation of RCe007-A (RC-3) to ectoderm (β-tubulin III), mesoderm (muscle actin), and endoderm (α-fetoprotein). Specific staining shown in green, cell nuclei are counterstained with DAPI (blue).

Table 2
Microsatellite PCR, blood group and HLA tissue typing results for RCe009-A (RC-5).

Microsatellite PCR results							
D3S1358 1 18	D3S1358 2 18	vWA 1 16	vWA 2 17	D16S539 1 9	D16S539 2 11	D2S1338 1 17	D2S1338 2 21
Amelogenin 1 X	Amelogenin 2 X	D8S1179 1 13	D8S1179 2 14	D21S11 1 29	D21S11 2 30	D18S51 1 10	D18S51 2 15
D19S433 1 14.2	D19S433 2 15	THO1 1 8	THO1 2 9	FGA 1 21	FGA 2 25	CSF1PO 1 10	CSF1PO 2 10
D5S818 1 11	D5S818 2 12	D7S820 1 10	D7S820 2 11	D13S317 1 8	D13S317 2 13	TPOX 1 8	TPOX 2 11

Blood group genotyping							
RhD pos	RhC neg	Rhc pos	RhE pos	Rhe pos	Fy a pos	Fy b pos	Fy GATA neg
Jka neg	Jkb pos	K neg	k pos	M pos	N pos	S neg	S pos
Kp a neg	Kp b pos	Do a neg	Do b pos	ABO AO1			

HLA Tissue Typing	
HLA Class I Type	HLA-A*01, A*32; B*40, B*57; C*02, C*06
HLA Class II Type	HLA-DRB1*13; DRB3*02; DQB1*06
Comment	B*40 is expressed serologically as B61.

Genomic analysis

All outsourced assays were carried out under a Quality and Technical Agreement. DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Manchester, UK) according to manufacturer's recommendations and provided in recommended quantities to the service providers.

Microsatellite PCR, or Short Tandem Repeat analysis, was used to determine cell line identity and was carried out by Public Health England. A profile was obtained for the following core alleles: vWA, D16S539, Amelogenin, THO1, CSF1PO, D5S818, D75820, D135317 and TPOX.

Human Leukocyte Antigen (HLA) tissue typing was carried out by the Scottish National Blood Transfusion Service.

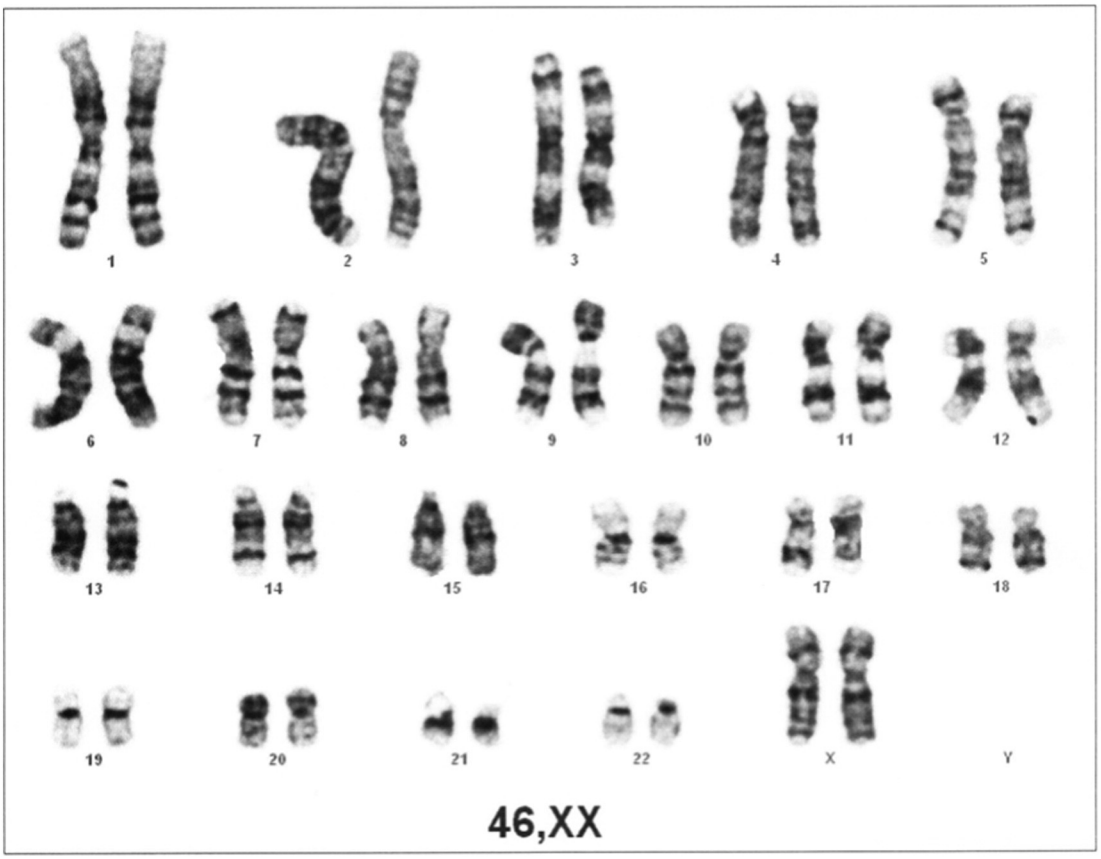

Fig. 4. RCe009-A (RC-5) was analysed by Giesma staining of 20 metaphase spreads and showed a normal 46XX female karyotype.

Blood group genotyping was carried out by the Molecular Diagnostics laboratory at NHSBT.

Karyotype analysis was carried out by The Doctors Laboratory (London, UK) or the Western General Cytogenetics Laboratory (Edinburgh, UK). Live cells at 60–70% confluency were shipped overnight in warm containers, fixed and analysed by standard G-banding analysis. For research grade lines, 20 spreads were analysed.

Acknowledgements

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